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8/27/99

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DATA EVALUATION RECORD

STUDY TYPE: Mammalian cells in culture gene mutation assay in Chinese hamster V79 cells
OPPTS Number: 870.5300 OPP Guideline Number: §84-2

DP BARCODE: D246707 SUBMISSION CODE: S543930
P.C. CODE: 112600 TOX. CHEM. NO.: None

TEST MATERIAL (PURITY): Prohexadione-calcium (93.3% a.i.)

SYNONYMS: Calcium-3,5-dioxo-4-propionyl-cyclohexane carboxylic acid

CITATION: Mullerschon, H. (1992) Gene Mutation Assay in Chinese Hamster V79 Cells In Vitro with Prohexadione-Calcium. Cytotest Cell Research GambH & Co. KG, D-6101 Rossdorf, Germany. CCR Project No. 304918, BASF Document Registration No. 92/11328. October 20, 1992. MRID 44499903. Unpublished

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EXECUTIVE SUMMARY:

In a mammalian cell gene mutation assay at the HGPRT locus (MRID 44499903), Chinese hamster V79 cells cultured in vitro were exposed in repeat (independent) assays to prohexadione-calcium (93.3% a.i.) at concentrations of 50, 100, 250, and 500 μg/mL in the absence of mammalian metabolic activation (-S9), and at concentrations of 50, 100, 250, and 475 μg/mL in the presence of S9 activation (+S9). Test cells were also exposed to ethylmethanesulfonate in culture medium (-S9; 600 μg/mL) or 7, 12-dimethylbenz(a)anthracene in DMSO (+S9; 3.85 μg/mL) for positive controls. Untreated culture medium served as the negative control (±S9), and a solvent control (DMSO) was included in the assay under S9 activation. Cultures were tested to the limit of solubility. The relative plating efficiencies at selection ranged from 104.3-112.8% without metabolic activation and from 76.6-103.9 % with metabolic activation.

Mutation frequencies were determined for concentrations selected on the basis of relative survival. In order for the test material to be considered a mutagen, the HGPRT forward mutant frequency had to be ≥3x that of the negative control at one dose level or show a reproducible, dose-dependent increase.

A reduced relative plating efficiency of 76.6% at the highest concentration of 475 μg/mL with

metabolic activation was observed in only one trial. There was neither a reproducible increase in mutation rate over the negative control at any treatment level nor a dose-related increase in the mutation rate with or without metabolic activation. **Therefore, under the conditions of the study, prohexadione-calcium was considered to be nonmutagenic in the presence or absence of S9 activation.** Under both the nonactivated and activated conditions, the positive controls induced the appropriate responses.

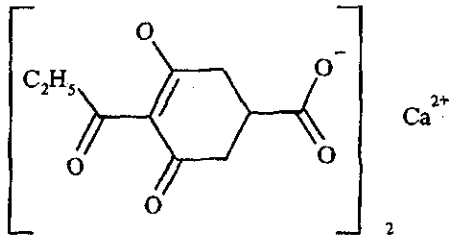
This study is classified as **acceptable (§84-2)**, and satisfies the guideline requirements for an *in vitro* forward gene mutation assay with mammalian activation.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Prohexadione-calcium
   Description: Bright yellow solid
   Lot/Batch #: FW 135
   Purity: 93.3% a.i.
   Stability of compound: Expiration date reported to be December 1992; stored at 4°C, protected from light. The study was conducted during July and August 1992.
   CAS #: 127277-53-6
   Structure:



   Solvent used: Culture medium (minimal essential medium, MEM)
   Other comments: The test substance was soluble in culture medium up to 500 μg/mL.

2. Control Materials:
   Negative: Untreated medium controls
   Solvent/final concentration: None
   Positive:
   Nonactivation: Ethylmethanesulfonate (EMS) at 0.6 mg/mL (4.8 mM) in culture medium

   Activation: 7,12-Dimethylbenz(a)anthracene (DMBA) at 3.85 μg/mL (15.0 μM) in dimethylsulfoxide (DMSO)

3. Activation: S9 derived from

_x_ Aroclor 1254	_x_ induced	_x_ rat	_x_ liver
__ phenobarbital	__ non-induced	__ mouse	__ lung
__ none		__ hamster	
__ other		__ other	

   If other, describe below

   Describe S9 mix composition (if purchased, give details): The S9 homogenate was

prepared in the testing laboratory and was derived from Aroclor 1254 induced male rat (strain Wistar/WU) liver. The S9 liver microsomal fraction was prepared by washing the livers removed from 8-12 week old rats with 150 mM KCl followed by homogenization and dilution of the homogenate 1:4 in KCl. The homogenate was centrifuged twice at 9,000 g/10 minutes, and 2 or 5 mL aliquots of the supematant were stored frozen at -70°C. A number of ampules were kept at -20°C for several weeks prior to use. Ampules of the S9 homogenate were thawed prior to use and mixed with S9 cofactor solution. The S9 cofactor solution consisted of 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, and 4 mM NADP in 100 mM sodium-ortho-phosphate buffer at pH 7.4. The S9 mix was stored in an ice bath until used. The final protein concentration of the S9 homogenate in culture medium was 0.75 mg/mL.

4. Test Cells: mammalian cells in culture
_ mouse lymphoma L5178Y cells
_ Chinese hamster ovary (CHO) cells
x V79 cells (Chinese hamster lung fibroblasts)
_ other (list):

Properly maintained? **Yes**
Periodically checked for Mycoplasma contamination? **Yes**
Periodically checked for karyotype stability? **Yes**
Periodically "cleansed" against high spontaneous background? **Yes**

Media: Growth medium was MEM medium supplemented with 10% fetal calf serum (FCS). During treatment, medium without serum was used. For selection of forward HGPRT mutants, the growth medium was supplemented with 11 µg/mL 6-thioguanine.

5. Locus Examined:
_ thymidine kinase (TK)
Selection agent: _____ bromodeoxyuridine (BrdU)
(give concentr.) _____ fluorodeoxyuridine (FdU)
_____ trifluorothymidine (TFT)

x hypoxanthine-guanine-phosphoribosyl transferase (HPRT)
Selection agent: _____ 8-azaguanine (8-AG)
(give concentr.) __x__ 6-thioguanine (6-TG), 11 µg/mL

_ Na^+/K^+ ATPase
Selection agent: _____ ouabain
(give concentr.)

_ other (locus and/or selection agent; give details):

6. Test compound concentrations used:

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a. Preliminary Cytotoxicity Assay:
Nonactivated and activated conditions: The concentration ranges used in the mutagenicity assays with and without metabolic activation were selected based on the results of a pre-experiment toxicity test. The toxicity test concentrations used were 0.10, 0.30, 1, 3, 10, 30, 100, 250, and 500 μg/mL with and without metabolic activation. The colony forming ability of approximately 500 single cells (duplicate cultures per concentration level) after treatment with the test substance was compared to the negative control. Toxicity was determined by reduction in plating efficiency (PE).

b. Mutation Assays: Two independent trials were initiated at four dose levels/condition carried to termination per trial.
Nonactivated conditions: 50, 100, 250, and 500 μg/mL
Activated conditions: 50, 100, 250, and 475 μg/mL

B. TEST PERFORMANCE

1. Cell treatment: Approximately 2 x 10^6 cells were seeded (1 flask per dose level) for mutagenicity testing and 500 cells were seeded (2 flasks per dose level) for cytotoxicity determination.

   a. Cells exposed to test compound, negative/solvent or positive controls for:
   4 hours (nonactivated) 4 hours (activated)

   b. After washing, cells for cytotoxicity determination were cultured for 6 days. After washing, cells for growth and expression of mutation were cultured for 3 days, then subcultured on day 5 and day 9.

   c. After expression, 3-5 x 10^5 cells/flask (5 flasks/ group) were cultured for 8 days in selection medium to determine numbers of mutants and 500 cells/flask (2 flasks/group) were cultured for 7 days without selective agent to determine plating efficiency.

2. Statistical Methods: Statistical analysis was not performed.

3. Evaluation Criteria: The assay was considered valid if (i) the numbers of mutant colonies per 10^6 cells found in the negative/solvent controls fall within the laboratory historical control data range of 5-45 mutants/10^6 cells; (ii) the positive control substances should produce a significant increase in mutant colony frequencies; (iii) the plating efficiency (absolute value) of the negative and/or solvent control should exceed 50%. The test material was considered mutagenic if (i) a reproducible, dose-related increase in mutant frequency was observed over a range of doses or (ii) the mutant frequency at one concentration exceeded that of the solvent control by a factor of ≥3 and was reproducible. However, in the case of a low spontaneous mutation rate (untreated and solvent controls) compared to historical controls (5-45 mutants/10^6 cells), an apparent dose-related increase in mutations up to a factor of 3 may be considered irrelevant.

II. **REPORTED RESULTS** 2.18

A. Analytical Determinations

The dosing solutions were not analyzed by the study laboratory for stability or actual concentration of test substance. The test material was soluble in culture medium at all dose levels evaluated with and without S9 activation (50-500 μg/mL).

B. Preliminary cytotoxicity assay

The results of the preliminary cytotoxicity assay are presented on page 22 of the study report. The cytotoxicity assay was conducted to determine the plating efficiency of Chinese hamster V79 cells treated with prohexadione-calcium with and without metabolic activation. The test concentrations used were 0.10, 0.30, 1, 3, 10, 30, 100, 250, and 500 μg/mL with and without metabolic activation. The plating efficiency of the V79 cells was weakly reduced after treatment with the highest concentration of 500 μg/mL with metabolic activation; the relative plating efficiency was 72.5%. Plating efficiency was not reduced at any dose level without metabolic activation. Based on these results, concentrations selected for the mutagenicity assay were 50, 100, 250, and 475 μg/mL for activation conditions, and 50, 100, 250, and 500 μg/mL for nonactivation conditions.

C. Mutagenicity assay:

The results of the mutagenicity and concurrent cytotoxicity assays are presented in study report Tables I-VI, pages 23-28). The results are compiled and summarized as mean revertant colonies per plate in Tables 1 and 2 below.

Nonactivation conditions: Two trials were performed at prohexadione-calcium treatment levels of 50, 100, 250, and 500 μg/mL. There were no significant or dose-related increases in mutation rate over negative controls at any treatment level. Relative survival was reduced by 12 to 33% compared to the negative control in the first trial; however, the reduction was inversely proportional to the dose.

Activation conditions: Two trials were performed at prohexadione-calcium treatment levels of 50, 100, 250, and 475 μg/mL. There were no significant increases in mutation rate over negative controls at any treatment level. A slightly decreased relative plating efficiency of 76.6% at selection was only observed at the highest concentration of 475 μg/mL in the first trial.

Table 1. Summary of Mutagenicity Assay Results - Trial 1

Test substance	Dose (μg/mL)	Relative Plating Efficiency (%)	Mutant colonies/10^6 cells	Relative survival (%)
Without metabolic activation (-S9)				
Negative control	0	100.0	10.8	100.0
EMS Positive control	600	48.9	528.3	73.7
Prohexadione-calcium	50	107.4	9.0	98.4
	100	112.8	9.8	86.0
	250	105.4	7.5	105.7
	500	107.2	7.7	90.8
With metabolic activation (+S9)				
Negative control	0	100.0	4.0	100.0
DMSO solvent control for DMBA	0	100.0	8.1	93.2
DMBA Positive control	3.85	18.0	550.8	75.1
Prohexadione-calcium	50	98.3	13.0	81.5
	100	101.7	13.4	98.1
	250	103.9	11.8	102.6
	475	76.6	6.0	93.4

Table 2. Summary of Mutagenicity Assay Results - Trial 2

Test substance	Dose (μg/mL)	Relative Plating Efficiency (%)	Mutant colonies/10^6 cells	Relative survival (%)
Without metabolic activation (-S9)				
Negative control	0	100.0	5.9	100.0
EMS Positive control	600	53.7	324.1	73.6
Prohexadione-calcium	50	111.6	5.0	66.8
	100	104.3	13.4	68.7
	250	104.8	18.9	70.9
	500	109.1	11.4	88.2
With metabolic activation (+S9)				
Negative control	0	100.0	8.2	100.0
DMSO solvent control for DMBA	0	100.0	5.0	112.1
DMBA Positive control	3.85	24.2	514.7	89.9
Prohexadione-calcium	50	99.1	3.8	124.3
	100	100.4	12.7	111.4
	250	93.6	12.1	114.1
	475	98.9	10.8	115.8

III. REVIEWER'S DISCUSSION/CONCLUSIONS:

A. Prohexadione-calcium was tested to the limit of solubility and was slightly cytotoxic at 500 μg/mL with metabolic activation. Two trials were conducted both with and without metabolic activation. There was no reproducible or significant increase in mutation rate at any dose level or condition compared to the negative control. The range of relative (to negative controls) plating efficiencies at selection with treatment ranged from 104.3-112.8% without metabolic activation and from 76.6-103.9% with metabolic activation. The reduced relative plating efficiency of 76.6% in the first trial at the highest concentration of 475 μg/mL was not reproducible. The positive controls induced the appropriate responses. There were three mean mutant frequencies that were ≥3x that of the negative control, one without metabolic activation and two with metabolic activation. However, all three occurred at the low or mid dose levels and were not reproducible. Under the conditions of these tests, prohexadione-calcium is not mutagenic in this forward mutation assay at the HGPRT locus.

B. Study deficiencies - Analyses were not performed to confirm the stability and actual

concentration of the dosing solutions. However, because prohexadione-calcium was tested to the limit of solubility, this deficiency is not expected to affect the interpretation of this HGPRT forward mutation assay. One other deficiency noted in the study that is not considered to affect the validity of the study results is that the final concentration of the solvent control (DMSO) for DMBA was not specified.